

Regulation of endoplasmic reticulum Ca^{2+} oscillations in mammalian eggs

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Summary

Changes in the intracellular concentration of free calcium ($[\text{Ca}^{2+}]_i$) regulate diverse cellular processes including fertilization. In mammalian eggs, the $[\text{Ca}^{2+}]_i$ changes induced by the sperm unfold in a pattern of periodical rises, also known as $[\text{Ca}^{2+}]_i$ oscillations. The source of Ca^{2+} during oscillations is the endoplasmic reticulum ($[\text{Ca}^{2+}]_{\text{ER}}$), but it is presently unknown how $[\text{Ca}^{2+}]_{\text{ER}}$ is regulated. Here, we show using mouse eggs that $[\text{Ca}^{2+}]_i$ oscillations induced by a variety of agonists, including PLC ζ , SrCl_2 and thimerosal, provoke simultaneous but opposite changes in $[\text{Ca}^{2+}]_{\text{ER}}$ and cause differential effects on the refilling and overall load of $[\text{Ca}^{2+}]_{\text{ER}}$. We also found that Ca^{2+} influx is required to refill $[\text{Ca}^{2+}]_{\text{ER}}$, because the loss of $[\text{Ca}^{2+}]_{\text{ER}}$ was accelerated in medium devoid of Ca^{2+} . Pharmacological inactivation of the function of the mitochondria and of the Ca^{2+} -ATPase pumps PMCA and SERCA altered the pattern of oscillations and abruptly reduced $[\text{Ca}^{2+}]_{\text{ER}}$, especially after inactivation of mitochondria and SERCA functions. We also examined the expression of SERCA2b protein and found that it was expressed throughout oocyte maturation and attained a conspicuous cortical cluster organization in mature eggs. We show that its overexpression reduces the duration of inositol-1,4,5-trisphosphate-induced $[\text{Ca}^{2+}]_i$ rises, promotes initiation of oscillations and enhances refilling of $[\text{Ca}^{2+}]_{\text{ER}}$. Collectively, our results provide novel insights on the regulation of $[\text{Ca}^{2+}]_{\text{ER}}$ oscillations, which underlie the unique Ca^{2+} -signalling system that activates the developmental program in mammalian eggs.

Key words: Ca^{2+} oscillations, Egg activation, Fertilization, Oocyte maturation, SERCA, Endoplasmic reticulum

Introduction

At fertilization, an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which is generally induced following interaction of the gametes, is the universal signal for the completion of meiosis and initiation of embryo development (Stricker, 1999). In mammals, this signal takes the form of periodical increases in $[\text{Ca}^{2+}]_i$, termed $[\text{Ca}^{2+}]_i$ oscillations, which last for several hours after sperm entry (Miyazaki et al., 1986). The $[\text{Ca}^{2+}]_i$ responses provide spatio-temporal information that is decoded by downstream effectors, whose actions underpin several distinct cellular events such as the release of cortical granules, progression into interphase and pronuclear formation (PN). These and other subtler events underlie the egg-to-embryo transition and are collectively known as ‘egg activation’ (Ducibella et al., 2002; Schultz and Kopf, 1995). Although $[\text{Ca}^{2+}]_i$ oscillations are a hallmark of mammalian fertilization and required for egg activation, the underlying molecular mechanism(s) that sustain them remain elusive.

Inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from the endoplasmic reticulum ($[\text{Ca}^{2+}]_{\text{ER}}$), the main Ca^{2+} reservoir of the cell (Berridge, 2002), is primarily responsible for the initial $[\text{Ca}^{2+}]_i$ wave and oscillations during fertilization (Miyazaki et al., 1992). Therefore, the general properties of Ca^{2+} release during fertilization have been described in the context of IP_3 production and regulation of its cognate receptor, IP_3R (Jellerette et al., 2000; Miyazaki, 1993). Nevertheless, the regulation of $[\text{Ca}^{2+}]_i$

oscillations in eggs or cells is likely to be far more complex, because oscillations require Ca^{2+} influx and clearing mechanisms. For instance, for $[\text{Ca}^{2+}]_i$ oscillations to continue without attenuation, eggs must replenish $[\text{Ca}^{2+}]_{\text{ER}}$, because in the absence of external Ca^{2+} ($[\text{Ca}^{2+}]_e$), sperm-initiated $[\text{Ca}^{2+}]_i$ oscillations run down and cease prematurely (Igusa and Miyazaki, 1983; Winston et al., 1995). Thus, $[\text{Ca}^{2+}]_e$ must cross the plasma membrane (PM) and access the ooplasm by a variety of channels and mechanisms (Smyth et al., 2006). Store-operated Ca^{2+} entry (SOCE) is one of these proposed mechanisms that is activated by the depletion of $[\text{Ca}^{2+}]_{\text{ER}}$ (Putney, 1990). However, excessive or prolonged $[\text{Ca}^{2+}]_i$ elevations abolish the oscillatory behavior, and can cause fragmentation and apoptosis (Gordo et al., 2002; Ozil et al., 2005). To prevent this, elevated $[\text{Ca}^{2+}]_i$ is rapidly removed by pumps and exchangers. The PM Ca^{2+} -ATPase (PMCA) and $\text{Na}^+/\text{Ca}^{2+}$ exchanger extrude free cytosolic Ca^{2+} to the extracellular space and the sarco-endoplasmic reticulum Ca^{2+} -ATPases (SERCA) reuptake it into the ER stores, thereby refilling them (Berridge et al., 2000; Bootman et al., 2001). The mitochondria also uptake cytosolic Ca^{2+} , thereby contributing to shape the spatio-temporal patterns of $[\text{Ca}^{2+}]_i$ responses, while simultaneously promoting a number of events that sustain ATP levels in cells (Hajnóczky et al., 1995). Despite the pivotal role of these mechanisms of Ca^{2+} homeostasis, few studies have examined their underlying molecules and regulation in mammalian oocytes and eggs.

In this regard, few factors are as important to sustain fertilization-associated $[\text{Ca}^{2+}]_i$ oscillations than $[\text{Ca}^{2+}]_{\text{ER}}$, because it is the source of Ca^{2+} during oscillations. Recent studies have shown that during fertilization, $[\text{Ca}^{2+}]_{\text{ER}}$ levels change in parallel with changes in $[\text{Ca}^{2+}]_i$ (Takahashi et al., 2013; Wakai and Fissore, 2013), although how $[\text{Ca}^{2+}]_{\text{ER}}$ levels are regulated during oscillations was not examined. Moreover, it is unknown whether common parthenogenetic agonists such as SrCl_2 or thimerosal, which reportedly induce $[\text{Ca}^{2+}]_i$ oscillations by sensitizing IP_3Rs and without generating IP_3 (Cheek et al., 1993; Galione et al., 1993), also modify $[\text{Ca}^{2+}]_{\text{ER}}$ levels. Hence, direct measurement of $[\text{Ca}^{2+}]_{\text{ER}}$ is required to get a better appreciation of Ca^{2+} homeostasis in these cells. Toward this end, advances in genetically encoded Ca^{2+} probes now allow the measurement of Ca^{2+} dynamics in live cells and in targeted organelles (Demaurex, 2005). Cameleon D1ER, a fluorescence resonance energy transfer (FRET)-based Ca^{2+} indicator, was successfully used to report $[\text{Ca}^{2+}]_{\text{ER}}$ in somatic cells (Palmer et al., 2004) and recently in mouse eggs (Takahashi et al., 2013; Wakai and Fissore, 2013).

Prior to ovulation and following a systemic LH surge, prophase-arrested germinal vesicle (GV) oocytes resume meiosis and progress to the metaphase stage of the second meiosis (MII). This process is commonly referred to as oocyte maturation, and during it, oocytes acquire fertilization-competence and the precise spatio-temporal pattern of fertilization-associated $[\text{Ca}^{2+}]_i$ responses. In fact, in-vitro-fertilized GV oocytes show fewer $[\text{Ca}^{2+}]_i$ oscillations and each $[\text{Ca}^{2+}]_i$ rise exhibits lesser duration and amplitude than those observed in fertilized MII oocytes (henceforth referred to as eggs) (Jones et al., 1995b; Mehlmann et al., 1996). Given that several of the parameters of Ca^{2+} homeostasis progressively change during maturation, including the steady increase of $[\text{Ca}^{2+}]_{\text{ER}}$ (Jones et al., 1995b; Mehlmann and Kline, 1994; Wakai et al., 2012), it is possible that the molecules responsible for these adjustments in Ca^{2+} homeostasis, which are mostly unknown in mammalian oocytes, experience dynamic modifications such that some of the mechanisms active at the GV stage might not be so at the MII stage and vice versa.

In the present study, using D1ER and mouse oocytes and eggs we measured $[\text{Ca}^{2+}]_{\text{ER}}$ levels during $[\text{Ca}^{2+}]_i$ oscillations induced by a variety of agonists. We found simultaneous changes in $[\text{Ca}^{2+}]_{\text{ER}}$ and $[\text{Ca}^{2+}]_i$ levels during oscillations, although with unique $[\text{Ca}^{2+}]_{\text{ER}}$ kinetics according to the agonist applied. Furthermore, we show that $[\text{Ca}^{2+}]_{\text{ER}}$ can act as a pacemaker of oscillations and is regulated by the action of mechanisms involved in Ca^{2+} influx and in buffering and sequestering $[\text{Ca}^{2+}]_i$. Finally, as well as showing that the SERCA2b protein is expressed in oocytes and eggs, we observed that it undergoes dynamic reorganization during maturation, which might contribute to shaping the fertilization-associated $[\text{Ca}^{2+}]_i$ responses in these cells.

Results

Agonist-dependent regulation of $[\text{Ca}^{2+}]_{\text{ER}}$ during $[\text{Ca}^{2+}]_i$ oscillations

We first examined whether expression of D1ER protein in both mouse oocytes and eggs attained a distribution that replicated that of the ER in these two stages of maturation. To accomplish this, D1ER cRNA was injected into GV oocytes and MII eggs, and fluorescence was examined ~5 hours after injection. Confocal

images showed that D1ER fluorescence displayed the widespread reticular pattern characteristic of the ER in these cell types, including the conspicuous cortical clusters localization distinctive of the ER in MII eggs (FitzHarris et al., 2007; Kline et al., 1999). Together, these results suggest that D1ER was successfully targeted to the ER of oocytes and eggs (Fig. 1A).

To explore the range of FRET signals in our system, which would allow estimating the relative changes in $[\text{Ca}^{2+}]_{\text{ER}}$ during Ca^{2+} stimulations, we recorded changes in emission ratios of D1ER (YFP/CFP) induced by addition of ionomycin. The studies were performed in the absence of $[\text{Ca}^{2+}]_e$, to maximize emptying of $[\text{Ca}^{2+}]_{\text{ER}}$ and prevent Ca^{2+} influx. Following addition of ionomycin, the emission ratio of D1ER immediately decreased, because the CFP signal increased and the YFP signal decreased (Fig. 1B). In the absence of agonists, emission ratios remained unchanged for at least 3 hours, although they gradually decreased during additional measurement (data not shown). To establish whether the change in $[\text{Ca}^{2+}]_{\text{ER}}$ closely corresponded with that of $[\text{Ca}^{2+}]_i$ levels, we performed simultaneous measurements of

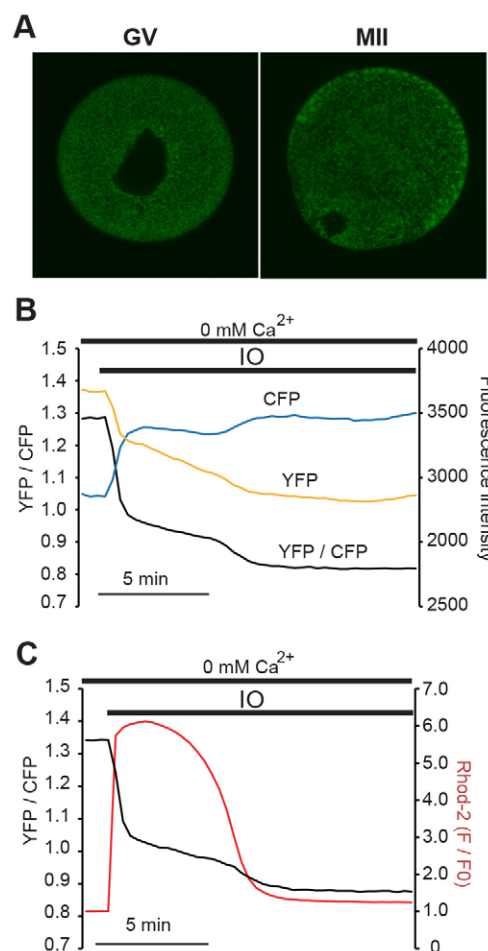


Fig. 1. Measurement of $[\text{Ca}^{2+}]_{\text{ER}}$ in oocytes and eggs using cameleon D1ER. (A) Confocal images of D1ER fluorescence in GV oocytes and MII eggs. (B,C) Emission ratios of D1ER (YFP/CFP; left axis) after addition of 5 μM ionomycin (IO) in Ca^{2+} -free medium. The intensities of CFP fluorescence and YFP fluorescence shifted in opposite directions (B). To perform simultaneous measurements of $[\text{Ca}^{2+}]_{\text{ER}}$ and $[\text{Ca}^{2+}]_i$, the latter was recorded using Rhod-2 (red trace, right axis) (C).

$[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_i$ using Rhod-2 as the Ca^{2+} indicator (Fig. 1C). Rhod-2 is generally used to measure mitochondrial Ca^{2+} , although given that in mouse eggs Rhod-2 fails to target to the mitochondria, it can be used to report cytoplasmic $[Ca^{2+}]_i$ (Dumollard et al., 2004). As expected, following addition of ionomycin, $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_i$ underwent simultaneous but opposite changes in relative concentrations.

We next examined changes in $[Ca^{2+}]_{ER}$ levels during $[Ca^{2+}]_i$ oscillations. $[Ca^{2+}]_i$ oscillations during mammalian fertilization are thought to be triggered following fusion of the gametes by release from the sperm of a male-specific phospholipase C (PLC) enzyme, PLC ζ (Nomikos et al., 2013; Saunders et al., 2002). Therefore, we initiated oscillations by injecting PLC ζ cRNA, which evokes $[Ca^{2+}]_i$ responses that closely and reproducibly resemble fertilization-induced $[Ca^{2+}]_i$ oscillations (Saunders et al., 2002). The results show that during oscillations the sharp upstroke of each $[Ca^{2+}]_i$ rise coincided with an equally fast drop in $[Ca^{2+}]_{ER}$ levels, as reported by a change in the YFP/CFP ratio (Fig. 2A). Remarkably, it appears that each of the first ~6–10 $[Ca^{2+}]_i$ rises occurred before $[Ca^{2+}]_{ER}$ levels had recovered to levels similar to those prior to the rise, which resulted in a progressive downturn of $[Ca^{2+}]_{ER}$ levels (Fig. 2B,C). In addition, the transient recovery of $[Ca^{2+}]_{ER}$ levels exhibited a two-step process, an initial rapid increase followed by a more gradual increase. Afterwards, basal $[Ca^{2+}]_{ER}$ stabilized and each $[Ca^{2+}]_i$ rise seemed to occur from the same $[Ca^{2+}]_{ER}$ level (Fig. 2D), which suggests that by then, the rate of refilling of the stores determines the initiation of the next $[Ca^{2+}]_i$ response.

$[Ca^{2+}]_i$ oscillations in eggs can also be induced by a variety of pharmacological agents, including strontium chloride ($SrCl_2$) and thimerosal. Nevertheless, the precise mechanism whereby these agents promote oscillations or the impact on $[Ca^{2+}]_{ER}$ levels are unknown. We first examined the role of $SrCl_2$, because it is widely used to induce artificial activation of mouse eggs

following somatic cell nuclear transfer or round spermatid injection (Loren and Lacham-Kaplan, 2006; Wakayama et al., 1998). To accomplish oscillations, medium devoid of $CaCl_2$ was supplemented with $SrCl_2$; this treatment generates repetitive $[Ca^{2+}/Sr^{2+}]_i$ rises that are characteristically of longer duration than those induced by other procedures (Fig. 3A). Remarkably, although a decrease in the overall $[Ca^{2+}]_{ER}$ levels was noticeable, the magnitude of the YFP/CFP ratio change was small (approximately less than half of the change observed in PLC ζ cRNA-induced $[Ca^{2+}]_i$ oscillations) and, in actuality, after a few $[Ca^{2+}/Sr^{2+}]_i$ rises, the levels of $[Ca^{2+}]_{ER}$ started to creep up, suggesting that oscillations by $SrCl_2$ rely less on intracellular $[Ca^{2+}]_i$ mobilization. Supporting this notion, it is worth noting that the changes in $[Ca^{2+}]_{ER}$ levels were slow and clearly behind the changes in $[Ca^{2+}]_i$, because by the time $[Ca^{2+}/Sr^{2+}]_i$ had peaked, $[Ca^{2+}]_{ER}$ had not changed (Fig. 3A, right panel). The $[Ca^{2+}]_i$ rises induced by thimerosal, a thiol-oxidizing agent known to induce oscillations in mammalian oocytes (Swann, 1991), caused greatly different effects on $[Ca^{2+}]_{ER}$ levels. For example, the first and subsequent $[Ca^{2+}]_i$ rises were not accompanied by a reduction but rather by an increase in $[Ca^{2+}]_{ER}$ levels (Fig. 3B). Further, in accordance with the high frequency of the oscillations, $[Ca^{2+}]_{ER}$ was rapidly refilled and eventually overloaded, as demonstrated by higher $[Ca^{2+}]_{ER}$ levels at the end of the monitoring period (Fig. 3B, right panel). Collectively, our results show that monitoring $[Ca^{2+}]_{ER}$ reveals specific agonist regulation of $[Ca^{2+}]_{ER}$, which is consistent with the unique pattern of oscillations by these agonists.

Ca^{2+} influx is required for replenishment of $[Ca^{2+}]_{ER}$ during $[Ca^{2+}]_i$ oscillations

To gain insight into the contribution of Ca^{2+} influx to the refilling of $[Ca^{2+}]_{ER}$, we simultaneously measured $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_i$ in eggs induced to oscillate by injection of PLC ζ cRNA while they

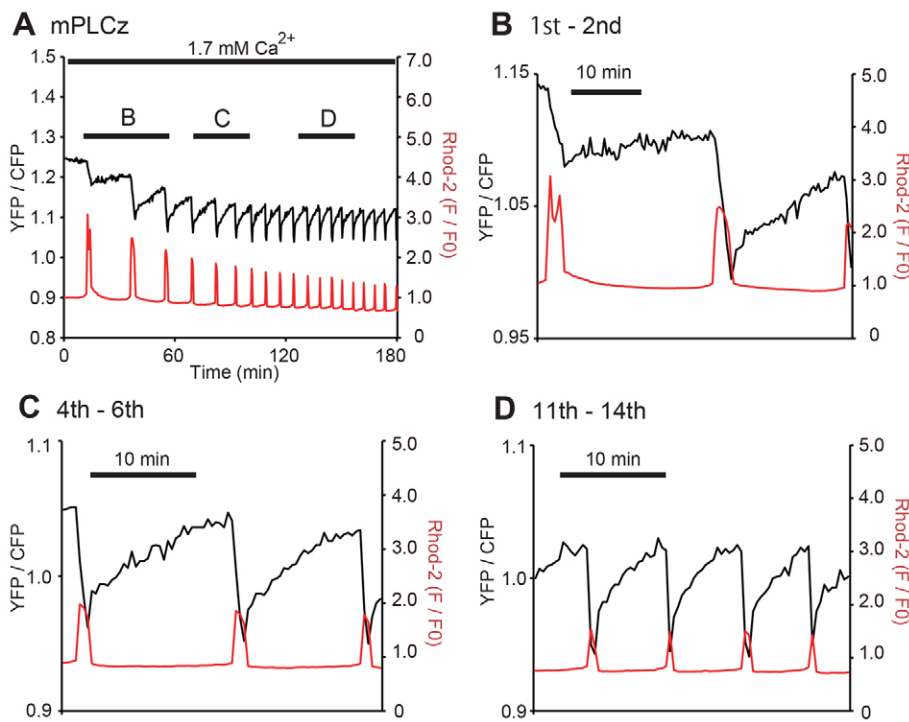


Fig. 2. $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_i$ responses during PLC ζ -induced oscillations. (A–D) Representative Ca^{2+} responses induced by injection of mouse PLC ζ cRNA (0.05 μ g/ μ l) ($n=18$). $[Ca^{2+}]_{ER}$ (black trace, left axis) and $[Ca^{2+}]_i$ (red trace, right axis) undergo simultaneous but opposite changes in concentration during oscillations (A). (B–D) Magnified views of first and second (B), fourth to sixth (C) and eleventh to fourteenth Ca^{2+} responses induced by injection of PLC ζ cRNA.

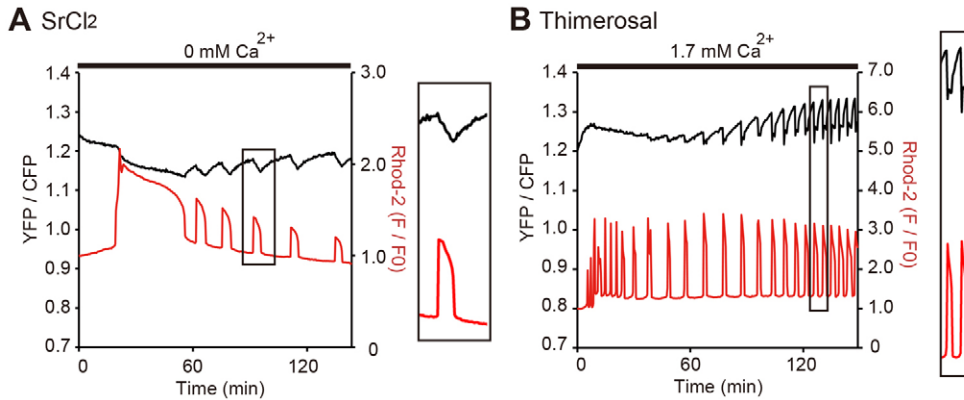


Fig. 3. $[\text{Ca}^{2+}]_{\text{ER}}$ and $[\text{Ca}^{2+}]_{\text{i}}$ during Sr^{2+} - and thimerosal-induced oscillations. (A,B) Simultaneous measurements of changes in $[\text{Ca}^{2+}]_{\text{ER}}$ and $[\text{Ca}^{2+}]_{\text{i}}$ in eggs exposed to 10 mM SrCl_2 ($n=16$) (A) or 50 μM thimerosal ($n=20$) (B). Right panels are magnified views of boxed areas on the main traces.

were maintained in Ca^{2+} -free conditions. As expected, $[\text{Ca}^{2+}]_{\text{i}}$ oscillations ceased prematurely and, on average, eggs only displayed 2.2 ± 0.32 $[\text{Ca}^{2+}]_{\text{i}}$ rises, supporting the notion that Ca^{2+} influx is required to maintain oscillations (Fig. 4A). $[\text{Ca}^{2+}]_{\text{ER}}$ levels also declined, which was confirmed by addition of ionomycin after the oscillations had ceased, because both $[\text{Ca}^{2+}]_{\text{i}}$ and $[\text{Ca}^{2+}]_{\text{ER}}$ responses were greatly reduced in PLC ζ -injected eggs compared with untreated controls cultured in Ca^{2+} -free medium (Fig. 4B). Furthermore, the absence of $[\text{Ca}^{2+}]_{\text{e}}$ slowed down the refilling of $[\text{Ca}^{2+}]_{\text{ER}}$ during oscillations, as reflected by the lower mean slope of the second $[\text{Ca}^{2+}]_{\text{ER}}$ increases in these eggs compared with those oscillating in the presence of $[\text{Ca}^{2+}]_{\text{e}}$ (Fig. 4C; Fig. 2A compare with Fig. 4A), which prolonged the interval between the first and second $[\text{Ca}^{2+}]_{\text{i}}$ rises in these eggs (Fig. 4D; Fig. 2A compare with Fig. 4A). The absence of $[\text{Ca}^{2+}]_{\text{e}}$ also compromised the duration of the first rise (Fig. 4E; Fig. 2A compare with Fig. 4A), confirming that Ca^{2+} influx also contributes to the robust first $[\text{Ca}^{2+}]_{\text{i}}$ rise (Halet et al., 2004). Collectively, our results therefore show that Ca^{2+} influx is required to replenish $[\text{Ca}^{2+}]_{\text{ER}}$ and shape $[\text{Ca}^{2+}]_{\text{i}}$ oscillations, because without it, $[\text{Ca}^{2+}]_{\text{i}}$ oscillations cease prematurely and show abnormal parameters.

Ca^{2+} -buffering mechanisms modulate refilling of $[\text{Ca}^{2+}]_{\text{ER}}$ during $[\text{Ca}^{2+}]_{\text{i}}$ oscillations

Compared with the quick return to baseline that cytosolic $[\text{Ca}^{2+}]_{\text{i}}$ transients experience during oscillations, the recovery of $[\text{Ca}^{2+}]_{\text{ER}}$ is more gradual, suggesting that other Ca^{2+} -buffering systems contribute to cytosolic $[\text{Ca}^{2+}]_{\text{i}}$ clearance. We therefore investigated the role of PMCA, one of the mechanisms known to mediate Ca^{2+} efflux, on the refilling of $[\text{Ca}^{2+}]_{\text{ER}}$. To accomplish this, we took advantage of the knowledge that PMCA is inhibited by millimolar concentrations of gadolinium (Gd^{3+}), which at these concentrations generates a Ca^{2+} insulation system, because both Ca^{2+} influx and efflux are greatly reduced (Bird and Putney, 2005); this approach was used in mouse eggs to maintain $[\text{Ca}^{2+}]_{\text{i}}$ oscillations in the absence of $[\text{Ca}^{2+}]_{\text{e}}$ (Miao et al., 2012). Using this experimental system (5 mM Gd^{3+}), we found that although the initiation of periodical oscillations was delayed by Gd^{3+} , injection of PLC ζ cRNA induced long-lasting $[\text{Ca}^{2+}]_{\text{i}}$ oscillations that were unattenuated despite the absence of $[\text{Ca}^{2+}]_{\text{e}}$ (Fig. 5A). Consistent with the high frequency of $[\text{Ca}^{2+}]_{\text{i}}$ rises, $[\text{Ca}^{2+}]_{\text{ER}}$ was rapidly refilled, and unlike $[\text{Ca}^{2+}]_{\text{i}}$ oscillations under normal $[\text{Ca}^{2+}]_{\text{e}}$, basal $[\text{Ca}^{2+}]_{\text{ER}}$ levels remained largely unchanged. These results suggest that PMCA shapes the pattern of $[\text{Ca}^{2+}]_{\text{i}}$

oscillations and inhibits its function, leading to retention of Ca^{2+} in the cytosol, which increases the refilling of $[\text{Ca}^{2+}]_{\text{ER}}$ by SERCA.

Mitochondrial function is closely linked to Ca^{2+} homeostasis, because inhibition of its function in mouse eggs disrupts $[\text{Ca}^{2+}]_{\text{i}}$ oscillations and causes an increase in $[\text{Ca}^{2+}]_{\text{i}}$ (Dumollard et al., 2004; Liu et al., 2001). To ascertain whether inhibition of mitochondrial function involved ER Ca^{2+} homeostasis, we exposed oscillating eggs to an inhibitor of mitochondrial ATP synthase (Wolvetang et al., 1994) and monitored the effects on the oscillations and $[\text{Ca}^{2+}]_{\text{ER}}$ refilling. Addition of oligomycin reduced $[\text{Ca}^{2+}]_{\text{ER}}$ levels, impaired its refilling and induced a sustained elevation in $[\text{Ca}^{2+}]_{\text{i}}$ in the majority of eggs (Fig. 5B). We interpret these results to mean that without ATP synthesis, the refilling of $[\text{Ca}^{2+}]_{\text{ER}}$ is compromised and oscillations cannot be maintained.

SERCA is required for replenishment of $[\text{Ca}^{2+}]_{\text{ER}}$ during $[\text{Ca}^{2+}]_{\text{i}}$ oscillations

The active presence of SERCA proteins in mammalian eggs can be surmised by the alteration of $[\text{Ca}^{2+}]_{\text{i}}$ levels caused by their exposure to thapsigargin or cyclopiazonic acid (CPA), two SERCA inhibitors; these inhibitors also prevent continuation of $[\text{Ca}^{2+}]_{\text{i}}$ oscillations in oscillating eggs (Kline and Kline, 1992; Lawrence and Cuthbertson, 1995; Macháty et al., 2002). Nevertheless, the precise rate of change of $[\text{Ca}^{2+}]_{\text{ER}}$ caused by SERCA inhibition has not been elucidated in these cells. We found that addition of thapsigargin to oscillating eggs immediately reduced basal $[\text{Ca}^{2+}]_{\text{ER}}$ levels and prevented their recovery thereafter (Fig. 5C). Thus, the results demonstrate that SERCA function is required for long-lasting $[\text{Ca}^{2+}]_{\text{i}}$ oscillations by maintaining $[\text{Ca}^{2+}]_{\text{ER}}$.

SERCA2b is expressed throughout oocyte maturation and undergoes spatial reorganization

Despite their pivotal role in Ca^{2+} homeostasis, the molecular properties of SERCA proteins and the dynamic modifications that they might undergo during maturation have not yet been examined in mammalian eggs. Three different SERCA genes (*ATP2A1–ATP2A3*) encode three main isoforms (*SERCA1–SERCA3*), each of which undergoes tissue-specific splicing (Brini and Carafoli, 2011). *SERCA1a* and *SERCA1b* are expressed in skeletal muscle, *SERCA2a* is found in cardiac muscle, whereas *SERCA2b* is ubiquitous and considered the housekeeping isoform. *SERCA3* is instead expressed in a limited

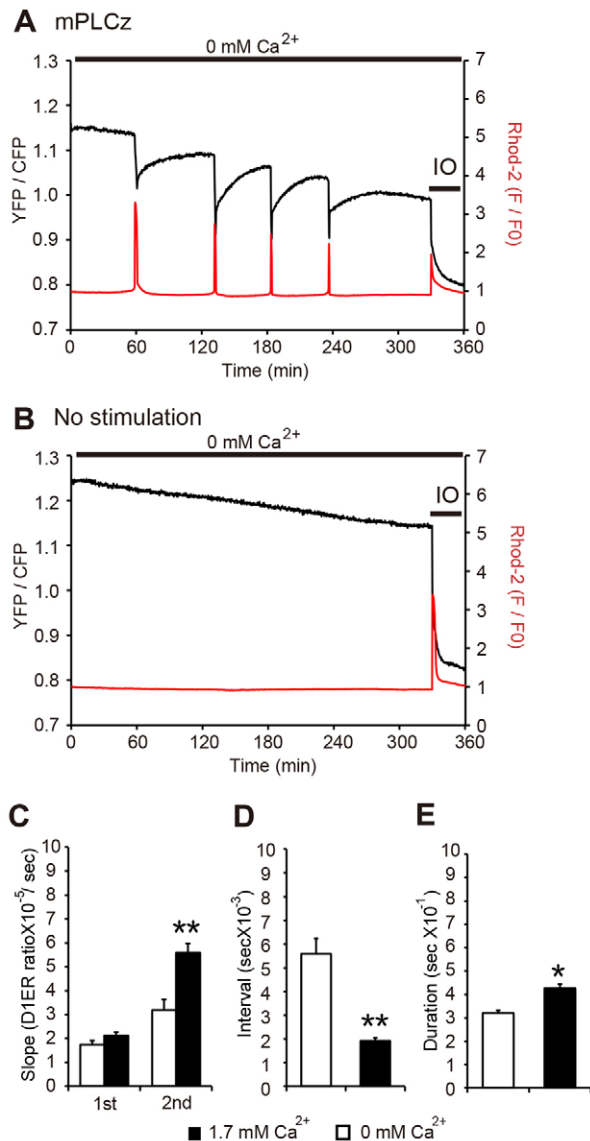


Fig. 4. $[\text{Ca}^{2+}]_{\text{ER}}$ and $[\text{Ca}^{2+}]_{\text{i}}$ responses induced by PLC ζ cRNA injection in the absence of $[\text{Ca}^{2+}]_{\text{e}}$. (A,B) Representative traces of Ca^{2+} responses induced by mouse PLC ζ cRNA injection ($n=20$) (A) or in uninjected controls ($n=5$) (B) in eggs maintained in Ca^{2+} -free conditions. $[\text{Ca}^{2+}]_{\text{ER}}$ and $[\text{Ca}^{2+}]_{\text{i}}$ were monitored as in previous experiments. When oscillations ceased, 2 μM ionomycin was applied. (C–E) Comparisons of several parameters of $[\text{Ca}^{2+}]_{\text{i}}$ rises of PLC ζ cRNA injection-induced oscillations in eggs oscillating in Ca^{2+} -containing ($n=17$) or in Ca^{2+} -free-medium ($n=20$). The recovery of $[\text{Ca}^{2+}]_{\text{ER}}$ after the first and second $[\text{Ca}^{2+}]_{\text{i}}$ transients was estimated by comparing the slope of refilling and plotted as changes in emission ratios of D1ER per second (C), the interval between first and second $[\text{Ca}^{2+}]_{\text{i}}$ rises (sec) (D) and duration of first $[\text{Ca}^{2+}]_{\text{i}}$ rise (F). Error bars represent s.e.m. * $P<0.005$; ** $P<0.001$.

number of non-muscle cells. We thus investigated the expression of SERCA2b protein and its subcellular distribution during *in vitro* maturation of oocytes.

Western blot analysis revealed that SERCA2b protein is expressed throughout maturation and comparable levels of protein are also found in *in vivo* matured MII eggs, as estimated by the intensity of SERCA2b-reactive bands

(artificial units) at different stages of maturation or after ovulation that showed values of 32.9, 39.6, 39.4, 44.6, 40.6 and 41.4 for oocytes at 0, 2, 4, 8, 12 hours after initiation of *in vitro* maturation and 14 hours after administration of hCG (*in vivo*), respectively (Fig. 6). To examine the subcellular distribution of SERCA2b, we used EGFP-tagged fusion proteins. We found that SERCA2b is diffusely distributed in GV oocytes, whereas it accumulates around the chromosomes at the GV breakdown (GVBD) stage (Fig. 7A, top panel). With progression of maturation, during the transition from the metaphase of first meiosis (MI) to MII, SERCA2b migrates toward the cortical regions surrounding the meiotic spindle. Time-lapse imaging of single oocytes also demonstrated the reorganization of SERCA2b during maturation (supplementary material Movie 1). Given that SERCA2b is an ER-resident protein, we used ER-tagged DsRed cRNA to determine whether it colocalized with the ER. We found that SERCA2b displayed identical localization as ER-DsRed (Fig. 7A, middle panel). Importantly, expression of higher concentration of RNA (1 $\mu\text{g}/\mu\text{l}$) to attain expression levels of SERCA2b-EGFP comparable to endogenous SERCA2b (Fig. 7B, bottom panel) revealed that SERCA2b displayed dense accumulations or clusters in the subcortical areas of MII eggs (Fig. 7B, top panel), which were not observed in GV oocytes. Furthermore, these clusters were more prominent in *in vivo* matured MII eggs and curiously remained detectable after extrusion of the second polar body extrusion (2PB) but disappeared by the PN stage after fertilization and did not reappear at the subsequent embryonic stage (2-cell) (Fig. 7C).

Microtubules and microfilaments in a step-wise manner are responsible for the redistribution that the ER undergoes during oocyte maturation (FitzHarris et al., 2007). In accordance with the ER reorganization, inhibition of microtubule polymerization by colcemid abrogated the accumulation of SERCA2b around the chromosomes at the GVBD stage (Fig. 7D), but not the subsequent migration to the cortex. However, treatment with Latrunculin A, which depolymerizes actin microfilaments, did not inhibit migration of SERCA2b toward the GV or spindle area, but prevented its migration to the cortex. Taken together, these results suggest that SERCA2b moves along cytoskeletal tracks during oocyte maturation and is actively re-organized into the cortical area in preparation for fertilization.

SERCA2b overexpression enhances ER Ca^{2+} uptake in oocytes and eggs

The increase in $[\text{Ca}^{2+}]_{\text{ER}}$ during oocyte maturation is a well-documented phenomenon (Jones et al., 1995b; Mehlmann et al., 1996; Wakai et al., 2012) that is likely to contribute to the robust Ca^{2+} release during fertilization (Wakai et al., 2012). To address the functional importance of SERCA2b for ER Ca^{2+} homeostasis during maturation, we investigated whether enhanced SERCA2b expression altered $[\text{Ca}^{2+}]_{\text{i}}$ responses in oocytes and eggs. This overexpression caused a significant increase in $[\text{Ca}^{2+}]_{\text{ER}}$ in GV oocytes, because the amplitude of ionomycin-evoked Ca^{2+} release was higher than in control GV oocytes ($P<0.05$), albeit far lower than in control MII eggs (Fig. 8A); $[\text{Ca}^{2+}]_{\text{ER}}$ levels in MII eggs were not increased by SERCA2b overexpression. The contribution of SERCA2b to the increase in $[\text{Ca}^{2+}]_{\text{ER}}$ was further demonstrated by inhibiting SERCA2b function using the pharmacological inhibitor CPA, which prevented the increase of $[\text{Ca}^{2+}]_{\text{ER}}$ in control or SERCA2b-expressing MII eggs. It should be noted that oocytes overexpressing SERCA2b or

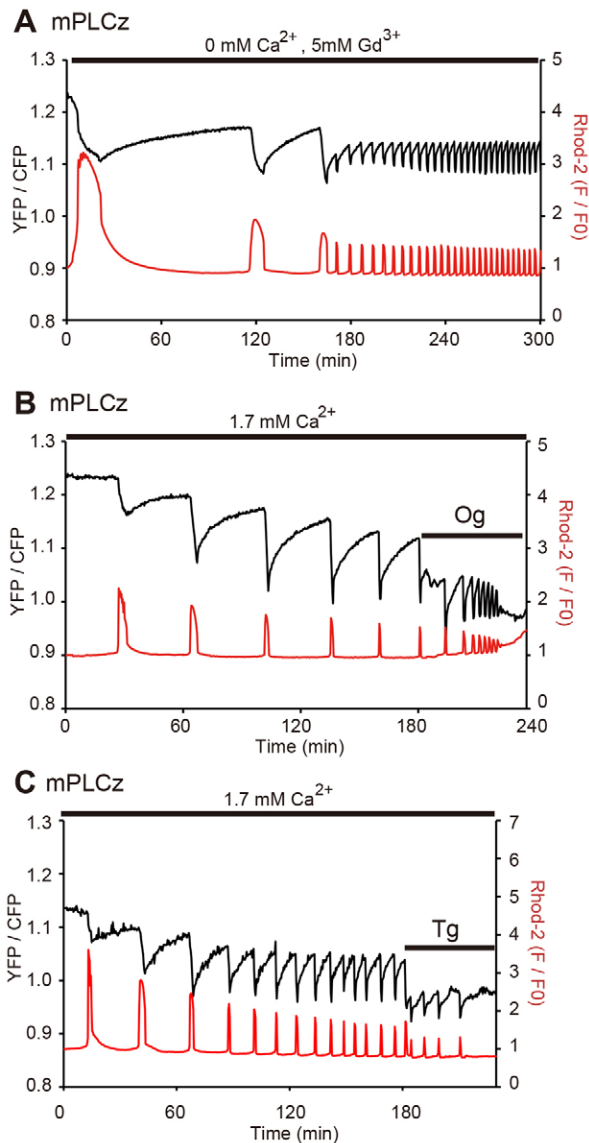


Fig. 5. Inhibition of the Ca^{2+} -buffering and Ca^{2+} -sequestering capacity of eggs alters $[\text{Ca}^{2+}]_{\text{ER}}$ and $[\text{Ca}^{2+}]_{\text{i}}$ responses during oscillations.

(A–C) Representative traces of the changes in $[\text{Ca}^{2+}]_{\text{ER}}$ (YFP/CFP; black trace) and $[\text{Ca}^{2+}]_{\text{i}}$ (Rhod-2; red trace) during PLC ζ -induced oscillations under conditions where Ca^{2+} efflux or influx ($n=22$) (A), mitochondrial function ($n=16$) (B) or SERCA ($n=18$) (C) were pharmacologically inactivated. (A) Simultaneous measurements were performed in Ca^{2+} -free HBSS medium containing 5 mM GdCl_3 . (B,C) Oligomycin and thapsigargin were added to oscillating eggs at concentrations of 5 μM and 20 μM , respectively.

matured in the presence of CPA advanced to the MII stage without complications, suggesting that the change in $[\text{Ca}^{2+}]_{\text{ER}}$ levels is dispensable for meiotic progression (Fig. 8B). Taken together, our results suggest that SERCA2b is required for the increase in $[\text{Ca}^{2+}]_{\text{ER}}$ during maturation.

We next examined the effects of SERCA2b overexpression on IP_3 -mediated Ca^{2+} release. To do this, IP_3 responses were produced by caged- IP_3 , which was photo-released by a flash of UV light as previously described (Wakai et al., 2012). To exclude the participation of Ca^{2+} influx, experiments were performed in Ca^{2+} -free medium. Consistent with our previous data, release of

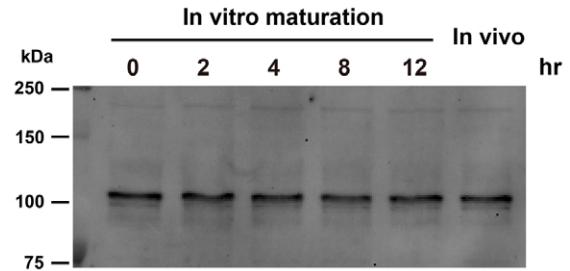


Fig. 6. SERCA2b is expressed throughout oocyte maturation. Western blot analysis of oocytes at different stages of maturation. Lysates of 100 oocytes were probed with an antibody specific for SERCA2b. A representative result of two similar independent experiments is shown.

IP_3 in control oocytes generated a single $[\text{Ca}^{2+}]_{\text{i}}$ rise that gradually returned to baseline (Fig. 8C). Nevertheless, in the majority of SERCA2b-overexpressing oocytes, although IP_3 release caused a similar $[\text{Ca}^{2+}]_{\text{i}}$ rise, it returned to basal levels considerably faster and displayed repetitive $[\text{Ca}^{2+}]_{\text{i}}$ transients before reaching baseline; time-lapse imaging shows the regenerative propagation of $[\text{Ca}^{2+}]_{\text{i}}$ responses (supplementary material Movie 2). Collectively, these results demonstrate that SERCA2b actively sequesters $[\text{Ca}^{2+}]_{\text{i}}$ during IP_3 -induced Ca^{2+} release.

Finally, we investigated whether SERCA2b overexpression influences $[\text{Ca}^{2+}]_{\text{ER}}$ homeostasis during oscillations. The overall patterns of $[\text{Ca}^{2+}]_{\text{i}}$ oscillations in SERCA2b-overexpressing eggs were indistinguishable from controls, because the duration, amplitude and frequency of $[\text{Ca}^{2+}]_{\text{i}}$ rises were not statistically different (data not shown). Nevertheless, after the fourth or fifth $[\text{Ca}^{2+}]_{\text{i}}$ rise, basal $[\text{Ca}^{2+}]_{\text{ER}}$ levels remained higher in SERCA2b-overexpressing eggs, suggesting that SERCA2b is one of the molecules that maintains $[\text{Ca}^{2+}]_{\text{ER}}$ during oscillations (Fig. 8D).

Discussion

Long-lasting $[\text{Ca}^{2+}]_{\text{i}}$ oscillations are a hallmark of mammalian fertilization and are important to establish the step-wise completion of all events of egg activation. The remarkable features presented here regarding Ca^{2+} homeostasis in mouse eggs include: (1) direct $[\text{Ca}^{2+}]_{\text{ER}}$ measurements demonstrate that refilling of $[\text{Ca}^{2+}]_{\text{ER}}$ underpins the sustained and periodical configuration of $[\text{Ca}^{2+}]_{\text{i}}$ oscillations; (2) the regulation of $[\text{Ca}^{2+}]_{\text{ER}}$ is closely associated with Ca^{2+} influx, Ca^{2+} buffering and sequestering mechanisms; (3) SERCA2b undergoes spatial redistribution during oocyte maturation, which is likely to optimize $[\text{Ca}^{2+}]_{\text{i}}$ responses during fertilization. Collectively, our results are the first to describe the regulation of $[\text{Ca}^{2+}]_{\text{ER}}$ in mammalian oocytes and set the stage to investigate how $[\text{Ca}^{2+}]_{\text{ER}}$ levels affect developmental competence.

Agonist-dependent $[\text{Ca}^{2+}]_{\text{ER}}$ filling and refilling during oscillations in mouse eggs

Understanding the regulation of $[\text{Ca}^{2+}]_{\text{ER}}$ is crucial to elucidate the mechanisms that underlie $[\text{Ca}^{2+}]_{\text{i}}$ oscillations. Using DIER, we show for the first time that $[\text{Ca}^{2+}]_{\text{ER}}$ undergoes agonist-specific patterns of oscillations. For example, PLC ζ -induced $[\text{Ca}^{2+}]_{\text{i}}$ oscillations triggered $[\text{Ca}^{2+}]_{\text{ER}}$ oscillations that displayed two notable characteristics: (1) basal $[\text{Ca}^{2+}]_{\text{ER}}$ levels progressively decreased during the initial $[\text{Ca}^{2+}]_{\text{i}}$ rises, although

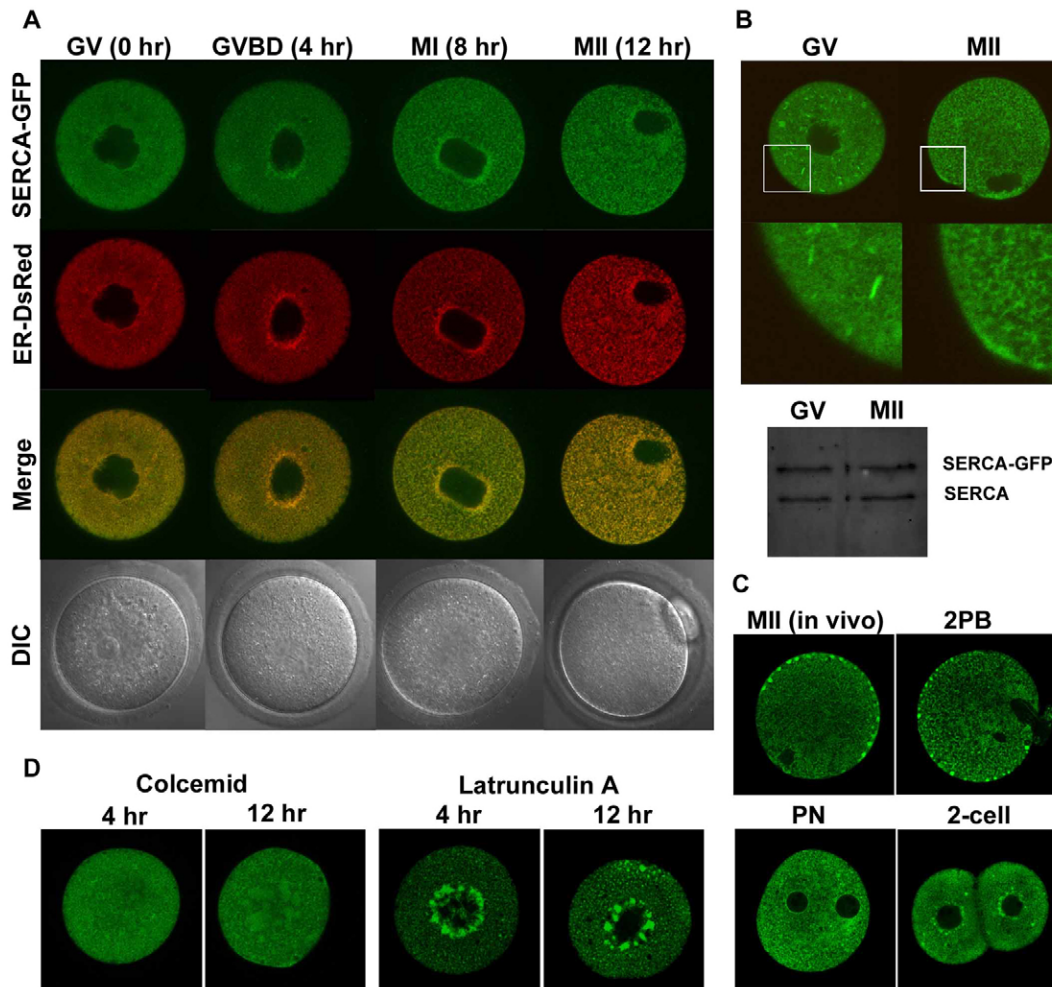


Fig. 7. SERCA2b undergoes reorganization during oocyte maturation and forms cortical clusters in MII eggs. (A) The subcellular distribution of SERCA2b and ER was analyzed using EGFP (top panel) and DsRed-tagged (middle panel) fusion proteins, respectively. Representative images taken at the equatorial plane are shown. The observations were performed at 0, 4, 8 and 12 hours after initiation of *in vitro* maturation, which corresponded with GV, GVBD, MI and MII stages, respectively; corresponding DIC images are shown in the bottom panel. (B) Images of high expression of SERCA2b-EGFP in GV oocytes and *in-vitro*-matured MII eggs (top panel), which was achieved by injection of 1 $\mu\text{g}/\mu\text{l}$ crRNA, and higher magnification views of the selected area (middle panel) where differences in SERCA2b distribution between the two stages can be observed. Levels of SERCA2b-EGFP expression were confirmed by western blot analysis (50 oocytes/eggs per lane) using antibody specific to SERCA2b (bottom panel). (C) Expression of SERCA2b-EGFP in *in-vivo*-matured MII eggs, 2PB or PN zygotes and two-cell embryo. (D) GV oocytes expressing SERCA2b-EGFP were matured in the presence of 100 ng/ml colcemid or 1 μM Latrunculin A and observations were performed at 4 and 12 hours of *in vitro* maturation; typical equatorial sections are shown.

they settled into steady state after certain time; (2) the recovery of $[\text{Ca}^{2+}]_{\text{ER}}$ after each $[\text{Ca}^{2+}]_{\text{i}}$ rise was accomplished in two phases, an initial rapid phase and a slower second phase. The progressive decrease in basal $[\text{Ca}^{2+}]_{\text{ER}}$ during initiation of oscillations might be explained at least in part by the large magnitude of the first rises coupled to the apparent inactivation of the Ca^{2+} influx mechanisms at the MII stage (Cheon et al., 2013). Furthermore, the presence of $[\text{Ca}^{2+}]_{\text{i}}$ oscillations despite decreasing $[\text{Ca}^{2+}]_{\text{ER}}$ levels might be associated with mouse eggs having a highly sensitized $\text{IP}_3\text{R1}$. MII eggs contain the highest $[\text{Ca}^{2+}]_{\text{ER}}$ levels of any stage, which, combined with increased ambient IP_3 induced by $\text{PLC}\zeta$, enhances $\text{IP}_3\text{R1}$ sensitivity, making Ca^{2+} release possible when $[\text{Ca}^{2+}]_{\text{ER}}$ is decreasing. In this context, it can be surmised that during these early stages of fertilization, the refilling of $[\text{Ca}^{2+}]_{\text{ER}}$ does not seem to set the pace of oscillations. Nevertheless, the decrease in $[\text{Ca}^{2+}]_{\text{ER}}$ eventually came to a halt

and thereafter $[\text{Ca}^{2+}]_{\text{ER}}$ levels became stable so that after each $[\text{Ca}^{2+}]_{\text{i}}$ rise $[\text{Ca}^{2+}]_{\text{ER}}$ levels fully recover in advance of the next $[\text{Ca}^{2+}]_{\text{i}}$ rise. This stabilization led to high synchrony between initiation of the upstroke of the $[\text{Ca}^{2+}]_{\text{i}}$ rise with initiation of the downstroke of $[\text{Ca}^{2+}]_{\text{ER}}$, which suggests that by this time the refilling of $[\text{Ca}^{2+}]_{\text{ER}}$ becomes the pacemaker of the oscillations. Moreover, at this point, Ca^{2+} efflux and influx is in balance with Ca^{2+} uptake and release from the ER. Concerning the refilling phases of $[\text{Ca}^{2+}]_{\text{ER}}$, the rapid phase appears to consist of Ca^{2+} reuptake from the cytosol by SERCA proteins, whereas the gradual phase might depend on Ca^{2+} influx from the external medium, because in the absence of $[\text{Ca}^{2+}]_{\text{e}}$, the latter was markedly extended.

In contrast to $\text{PLC}\zeta$ -induced oscillations, $[\text{Ca}^{2+}]_{\text{ER}}$ levels remain largely unchanged or even enhanced during Sr^{2+} - or thimerosal-induced oscillations. In the case of SrCl_2 , only the

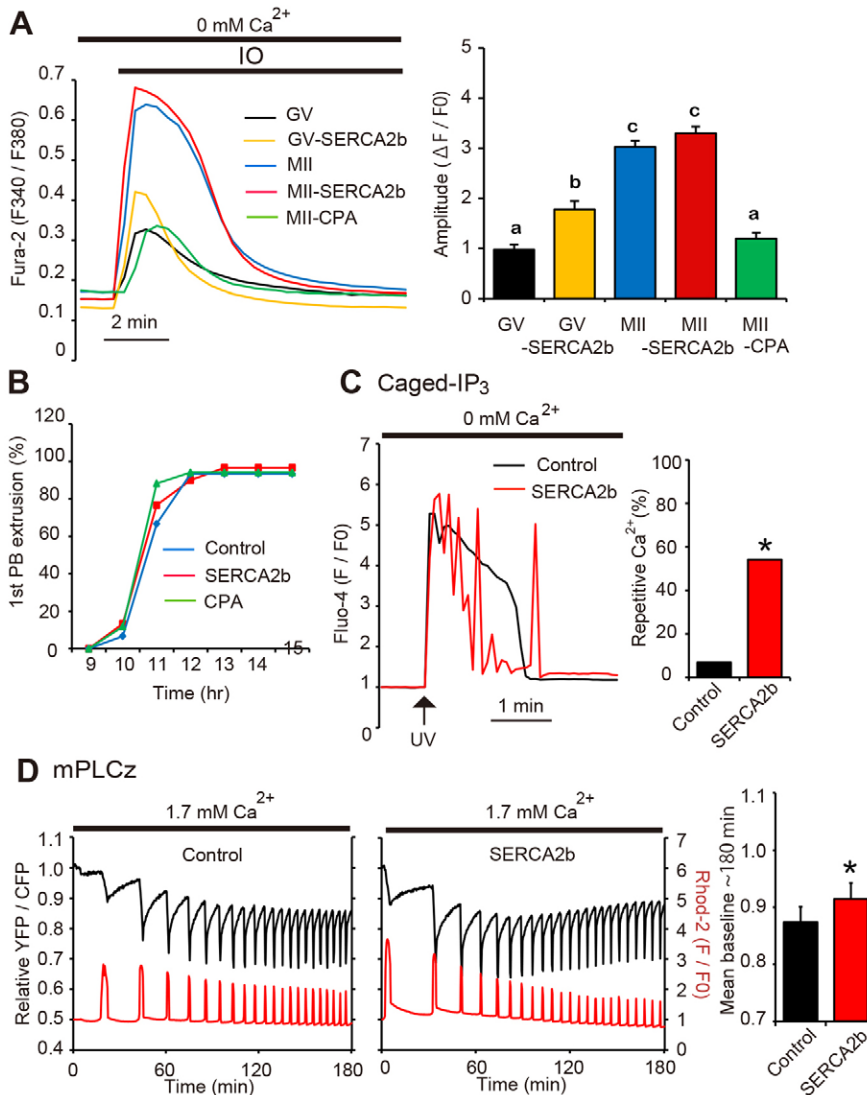


Fig. 8. SERCA2b overexpression alters Ca^{2+} responses in oocytes/eggs. (A) $[\text{Ca}^{2+}]_{\text{ER}}$ levels were estimated from $[\text{Ca}^{2+}]_{\text{i}}$ responses induced by addition of 2 μM ionomycin in Ca^{2+} free medium and representative traces of Fura-2 emission ratios are shown. The comparison of fluorescent $[\text{Ca}^{2+}]_{\text{i}}$ peaks between stages is shown in a bar graph to the right of the traces ($n=18-26$). Error bars represent s.e.m. and bars with different superscripts are significantly different ($P<0.05$). (B) Meiotic progression to the MII stage indicated as the percentage of oocytes with extrusion of the first polar body ($n=16-23$). (C) IP_3 -induced Ca^{2+} release obtained after photolysis of cIP₃ release (0.25 mM) by a flash of UV (arrow; 0.1 second). Representative traces of increases of Fluo-4 fluorescence caused by cIP₃ in control (black trace; $n=29$) and in oocytes overexpressing SERCA2b (red trace; $n=24$) are shown. The percentage of oocytes showing repetitive $[\text{Ca}^{2+}]_{\text{i}}$ rises were compared and displayed in a bar graph to the right. Asterisk indicates statistical significance ($*P<0.01$, Chi-squared test). (D) The relative emission ratio of D1ER (the value at the beginning of measurement was defined as 1; black line) and $[\text{Ca}^{2+}]_{\text{i}}$ (Rhod-2; red trace) during PLC ζ -induced oscillations were measured and representative traces are shown in control ($n=14$) and in SERCA2b-overexpressing eggs ($n=20$). Bar graphs show comparison of $[\text{Ca}^{2+}]_{\text{ER}}$ basal levels ~180 minutes after initiation of monitoring. $*P<0.05$, Student's *t*-test.

initial, prolonged rise, possibly caused by sensitization of $\text{IP}_3\text{R1}$ by SrCl_2 (Cheek et al., 1993; Zhang et al., 2005), led to a slow decline in $[\text{Ca}^{2+}]_{\text{ER}}$. After that, however, $\text{Ca}^{2+}/\text{Sr}^{2+}$ mobilization from the ER appeared to be modest, because by the time $[\text{Ca}^{2+}/\text{Sr}^{2+}]_{\text{i}}$ levels peaked, $[\text{Ca}^{2+}]_{\text{ER}}$ levels did not show a corresponding reduction, which suggests that Sr^{2+} from the external milieu is directly contributing to the cytosolic oscillations. Nevertheless, additional studies are needed to ascertain with higher degree of resolution the timing of $[\text{Ca}^{2+}]_{\text{i}}$ increases and $[\text{Ca}^{2+}]_{\text{ER}}$ decreases during oscillations. Furthermore, we are unaware of the ability of D1ER to bind Sr^{2+} and the affinity of SERCA for Sr^{2+} . Thimerosal produced the most striking results in $[\text{Ca}^{2+}]_{\text{ER}}$, because overall $[\text{Ca}^{2+}]_{\text{ER}}$ levels seemed to increase with progression of oscillations. Thimerosal has been proposed to initiate high-frequency $[\text{Ca}^{2+}]_{\text{i}}$ oscillations by sensitizing IP_3Rs (Cheek et al., 1993; McGuinness et al., 1996). Moreover, it has been speculated that by acting on cortical IP_3Rs , it might more effectively deplete peripheral stores, thereby promoting near-constitutive Ca^{2+} entry (McGuinness et al., 1996). This persistent Ca^{2+} entry might cause rapid saturation of the cytoplasmic stores, thereby promoting premature Ca^{2+}

release and reducing the interval between spikes. Thimerosal could also directly promote Ca^{2+} influx or reduce Ca^{2+} efflux, further contributing to the rapid refilling of $[\text{Ca}^{2+}]_{\text{ER}}$ and increased basal $[\text{Ca}^{2+}]_{\text{ER}}$ with progression of oscillations, as observed in our studies. Collectively, our results show that oscillations induced by different agonists cause distinct changes in $[\text{Ca}^{2+}]_{\text{ER}}$ levels in mouse eggs and Ca^{2+} influx-coupled $[\text{Ca}^{2+}]_{\text{ER}}$ refilling might control the frequency of oscillations.

Ca^{2+} influx and buffering mechanisms during oscillations

Accumulating evidence shows that Ca^{2+} influx is required for the persistence of $[\text{Ca}^{2+}]_{\text{i}}$ oscillations after fertilization (Lee et al., 2013; Lee et al., 2012; Shirakawa and Miyazaki, 1995; Wang et al., 2012). In the present study, we show that one role of Ca^{2+} influx is to replenish $[\text{Ca}^{2+}]_{\text{ER}}$ levels, because in the absence of $[\text{Ca}^{2+}]_{\text{e}}$ the rate of $[\text{Ca}^{2+}]_{\text{ER}}$ refilling was slowed, which led to the premature termination of PLC ζ -induced oscillations. Remarkably, by the time oscillations ceased in these eggs, $[\text{Ca}^{2+}]_{\text{ER}}$ levels were still substantial, because application of ionomycin caused a larger reduction in $[\text{Ca}^{2+}]_{\text{ER}}$ levels, which suggests that IP_3 -sensitive stores are a comparatively small part

of the total Ca^{2+} stored in the cell. Ca^{2+} -free medium also reduced the duration of the first $\text{PLC}\zeta$ -induced $[\text{Ca}^{2+}]_i$ rise, although it did not affect the $[\text{Ca}^{2+}]_{\text{ER}}$ recovery slope (Fig. 4C), which suggests that Ca^{2+} influx prolongs the first rise, possibly by causing Ca^{2+} -induced Ca^{2+} release. Ca^{2+} -free medium did not affect the $[\text{Ca}^{2+}]_{\text{ER}}$ recovery slope after the first $[\text{Ca}^{2+}]_i$ rise (Fig. 4E), suggesting that Ca^{2+} influx does not contribute to refill $[\text{Ca}^{2+}]_{\text{ER}}$ at this stage, which is consistent with the sharp loss of $[\text{Ca}^{2+}]_{\text{ER}}$ levels following this rise. Importantly, we are still unaware of the channels that mediate Ca^{2+} influx during maturation and fertilization, and thus, future studies should examine the type of plasma membrane channel(s) responsible for the influx.

The lag time between the rapid recovery of $[\text{Ca}^{2+}]_i$ and the slow refill of $[\text{Ca}^{2+}]_{\text{ER}}$ during oscillations suggests that additional Ca^{2+} -buffering mechanisms besides SERCA participate in returning $[\text{Ca}^{2+}]_i$ to basal levels. There are several possible mechanisms including the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which is known to be active in mouse eggs (Carroll, 2000; Pepperell et al., 1999), although here we focused on the roles of PMCA and the mitochondria, because mouse eggs maintained normal oscillatory patterns in Na^+ -free medium (Carroll, 2000). We used high concentrations of Gd^{3+} to examine the contribution of PMCA. In its presence, the duration of the initial $[\text{Ca}^{2+}]_i$ rises induced by injection of $\text{PLC}\zeta$ cRNA was increased and the interval between spikes widened. We interpret the broadening of the initial $[\text{Ca}^{2+}]_i$ rises to be due at least in part to the sensitization of $\text{IP}_3\text{R1}$ caused by the retention of Ca^{2+} in the cytosol and/or the enhanced SERCA-mediated Ca^{2+} reuptake, because the decline of $[\text{Ca}^{2+}]_{\text{ER}}$ levels was also greatly prolonged. The significant initial depletion of $[\text{Ca}^{2+}]_{\text{ER}}$ might require longer refilling, thereby prolonging the initial interspike intervals. Interestingly, ~3 hours after initiation of the $[\text{Ca}^{2+}]_i$ responses, the oscillations became very frequent and of small amplitude, which might be due to faster than normal Ca^{2+} reuptake into the ER and rapid refilling of $[\text{Ca}^{2+}]_{\text{ER}}$ caused by the modest $[\text{Ca}^{2+}]_i$ increases. Therefore, our results suggest active participation of PMCA in shaping the pattern of $[\text{Ca}^{2+}]_i$ oscillations and future studies should identify the molecular presence of PMCA in mouse eggs.

The mitochondria also contribute to shape $[\text{Ca}^{2+}]_i$ rises during oscillations (Duchen, 2000; Rizzuto et al., 2000), because they can uptake Ca^{2+} into the matrix, thereby alleviating the overall cytosolic Ca^{2+} load (Rizzuto et al., 1998). In support of this role, inhibition of mitochondrial function in mouse eggs disrupted oscillations and caused a sustained increase in $[\text{Ca}^{2+}]_i$ (Dumollard et al., 2004; Liu et al., 2001), although this effect was reportedly more related to the ability of mitochondria to produce ATP than their ability to uptake Ca^{2+} (Dumollard et al., 2004). Our results support this view, because inhibition of mitochondrial function with oligomycin reduced $[\text{Ca}^{2+}]_{\text{ER}}$ levels, inhibited refilling and terminated oscillations. The increase in basal $[\text{Ca}^{2+}]_i$ is possibly due to a malfunction of SERCA and PMCA pumps. Thus, mitochondrial function seems to be indispensable for $[\text{Ca}^{2+}]_i$ oscillations, because it is required both to maintain Ca^{2+} levels in the cytosol and in the ER through ATP-driven Ca^{2+} -pumping mechanisms.

The roles of SERCA proteins during oocyte maturation and fertilization

Despite the functional evidence that SERCA2b is associated with $[\text{Ca}^{2+}]_i$ oscillations in mouse eggs (Kline and Kline, 1992),

molecular evidence of SERCA2b and its contribution to the filling and refilling of $[\text{Ca}^{2+}]_{\text{ER}}$ in these cells has not been confirmed. Here, we show that inhibition of SERCA2b during maturation prevents the increase in $[\text{Ca}^{2+}]_{\text{ER}}$ during this process. Furthermore, we show that the protein remains uniformly expressed from the GV stage, which suggests that mechanisms other than protein expression must account for the increase in $[\text{Ca}^{2+}]_{\text{ER}}$ during maturation.

SERCA2b undergoes major cellular redistribution during maturation. The overall reorganization is similar to that described for the ER and IP_3Rs , both of which form marked clusters in subcortical areas in MII eggs. As a consequence of this reorganization, SERCA2b and $\text{IP}_3\text{R1}$ are closely apposed, thereby facilitating the refilling of ER Ca^{2+} stores rich in $\text{IP}_3\text{R1}$, which are probably frequently depleted during fertilization. It is worth noting that SERCA2b clusters remain past the 2PB stage, ~3 hours after fertilization, but disappear by the PN stage, ~8 hours after fertilization, coinciding with the natural duration of the $[\text{Ca}^{2+}]_i$ oscillations in these species (Jones et al., 1995a).

Our results also show that inhibition of SERCA2b by pharmacological inhibitors disrupts the refilling of $[\text{Ca}^{2+}]_{\text{ER}}$, lowering $[\text{Ca}^{2+}]_{\text{ER}}$ levels and causing premature termination of oscillations. Remarkably, despite lower $[\text{Ca}^{2+}]_{\text{ER}}$ levels, most eggs continued to oscillate for an additional 20–30 minutes, a finding previously noted by others, which suggests that mouse eggs contain thapsigargin-insensitive or -resistant stores that can support oscillations (Kline and Kline, 1992). The contribution of SERCA2b to oscillations was also addressed in overexpression studies. Expression of exogenous SERCA2b reduced the duration of $[\text{Ca}^{2+}]_i$ rises generated by caged IP_3 while at the same time stimulating the presence of oscillations. This result is consistent with data in *Xenopus* oocytes where SERCA overexpression increased the frequency of IP_3 -induced $[\text{Ca}^{2+}]_i$ oscillations and repetitive $[\text{Ca}^{2+}]_i$ rises were observed during the decaying phase of photo-release IP_3 -induced responses (Camacho and Lechleiter, 1993). We also observed that SERCA2b overexpression lessened the loss of $[\text{Ca}^{2+}]_{\text{ER}}$ levels experienced by eggs injected with mPLC ζ cRNA.

In conclusion, the present study provides novel insights into the function of $[\text{Ca}^{2+}]_{\text{ER}}$ during oscillations in mouse eggs, and shows that agonists have distinct impact on the refilling and overall load of $[\text{Ca}^{2+}]_{\text{ER}}$. We also found that Ca^{2+} influx and the function of Ca^{2+} -ATPase pumps and mitochondria are all key regulators of $[\text{Ca}^{2+}]_{\text{ER}}$ during maturation and fertilization. Future studies should identify the channels that regulate Ca^{2+} influx into oocytes and eggs and the regulatory mechanisms that modulate the function of the Ca^{2+} pumps and the mitochondria, because this knowledge will make it possible to manipulate $[\text{Ca}^{2+}]_{\text{ER}}$ levels to ascertain its impact on maturation and development.

Materials and Methods

Chemical reagents

Ionomycin, cyclopiazonic acid, thapsigargin, oligomycin and Latrunculin A were purchased from Calbiochem (San Diego, CA). Fura-2AM, Fluo-4AM, Rhod2AM, pluronic acid and caged- IP_3 (cIP $_3$) were purchased from Invitrogen (Carlsbad, CA). Other all chemicals were from Sigma (St Louis, MO) unless otherwise specified.

Collection of oocytes/eggs

GV oocytes and MII eggs were collected from the ovaries of 4- to 8-week-old and oviducts of 6- to 10-week-old CD-1 female mice, respectively. Females were injected with 5 IU pregnant mare serum gonadotropin (PMSG). Cumulus-cell-enclosed GV

oocytes were recovered 42–46 hours post-PMSG and the cumulus cells were removed by repeated pipetting. Ovulated MII eggs were recovered 12–14 hours after injection of 5 IU hCG, which was administered 44–48 hours after PMSG stimulation. All procedures were performed according to research animal protocols approved by the University of Massachusetts Institutional Animal Care and Use Committee.

Plasmids

Human SERCA2b was subcloned into a pcDNA6 vector (pcDNA6/Myc-His B; Invitrogen, Carlsbad, CA) between the *XhoI* and *PmeI* restriction sites. To analyze the subcellular distribution, SERCA2b was N-terminally tagged with EGFP between the *EcoRV* and *XhoI* restriction sites. Cameleon D1ER and pDsRed2-ER were kindly provided by R. Tsien (UCSD) and M. Trebak (Albany Medical College), respectively. The ER-targeting sequence of calreticulin, DsRed2 and the KDEL ER retention sequence were ligated to pcDNA6. Because the original D1ER construct somehow failed to target ER compartment in oocytes/eggs, the cameleon portion was amplified by PCR and inserted between calreticulin targeting and KDEL sequences in place of DsRed2. Mouse PLC ζ was a kind gift from K. Fukami (Tokyo University of Pharmacy and Life Science, Japan) and was subcloned into a PCS2+ vector, as previously described (Kurokawa et al., 2007).

Preparation and microinjection of cRNA

Plasmids were linearized with a restriction enzyme downstream of the insert to be transcribed. cDNA was *in vitro* transcribed using the T7 or SP6 mMESSAGE mMACHINE Kit (Ambion, Austin, TX) according to the promoter that is contained in the constructs. A poly(A) tail was added to the mRNAs using a Tailing Kit (Ambion) and poly(A)-tailed RNAs were eluted with RNase-free water and stored in aliquots at -80°C . For microinjection, cRNA solution was loaded into glass micropipettes and delivered into oocytes/eggs by pneumatic pressure (PLI-100, Harvard Apparatus, Cambridge, MA). Each oocyte received 7–12 pl (~ 1 –3% of the total volume of the egg). The volumes injected typically range from 2 to 10 pl, which is 1–5% of the egg (~ 70 –75 μm).

Ca^{2+} imaging

To estimate relative changes in $[\text{Ca}^{2+}]_{\text{ER}}$, emission ratio imaging of the D1ER (YFP/CFP) was performed using a CFP excitation filter, dichroic beamsplitter, CFP and YFP emission filters (Chroma technology, Rockingham, VT; ET436/20X, 89007bs, ET480/40m and ET535/30m). To measure $[\text{Ca}^{2+}]_{\text{ER}}$ and $[\text{Ca}^{2+}]_{\text{i}}$ simultaneously, eggs that had been injected with Cameleon D1ER cRNA were loaded with 1 μM Rhod-2AM supplemented with 0.02% pluronic acid for 20 minutes at room temperature. Eggs then were attached on glass-bottom dishes (MatTek Corp., Ashland, MA) and placed on the stage of an inverted microscope. CFP, YFP and Rhod-2 intensities were collected every 20 seconds by a cooled Photometrics SenSys CCD camera (Roper Scientific, Tucson, AZ). The rotation of excitation and emission filter wheels was controlled using the MAC5000 filter wheel/shutter control box (Ludl) and NIS-elements software (Nikon). Fura-2AM (1.25 μM) and Fluo-4AM (1 μM) were used to analyze ionomycin-induced and ClP_3 -induced Ca^{2+} rises, respectively. Fura-2 fluorescence was excited with 340 nm and 380 nm wavelengths every 20 seconds and emitted light was collected at wavelengths above 510 nm. Fluo-4 was excited with 480 nm wavelengths every 5 seconds and emitted light was collected at wavelengths greater than 510 nm. ClP_3 (0.25 mM) was injected into oocytes and the photolysis was accomplished with a 360 nm wavelength.

Confocal microscopy

Live-cell imaging of oocytes/eggs expressing fluorescent-tagged proteins was performed using a laser-scanning confocal microscope (LSM 510 META, Carl Zeiss Microimaging Inc., Germany) fitted with a 63×1.4 NA oil-immersion objective lens. Images were acquired with LSM software (Carl Zeiss Microimaging Inc., Germany) and pseudocolored in Photoshop CS (Adobe). Time-lapse imaging of SERCA2b-EGFP was performed using a Nipkow disk confocal unit (CV-1000, Yokogawa Electric Corp., Japan) outfitted with a $30\times$ silicone immersion objective lens.

Western blot analysis

Cell lysates from mouse oocytes/eggs were prepared by adding $2\times$ sample buffer. Proteins were separated by SDS-PAGE and transferred to PVDF membrane (Millipore, Bedford, MA). After blocking with 6% skimmed milk dissolved in TBS supplemented with 0.05% Tween-20 (TBST) for 2 hours at 4°C , membranes were probed with the rabbit polyclonal antibody specific to SERCA2b (Vangheluwe et al., 2007) (1:2000) in TBST with 3% skimmed milk overnight at 4°C . Goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) was used as a secondary antibody (1:2000, Bio-Rad, Hercules, CA) for detection of chemiluminescence (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions. The signal was digitally captured using a Kodak Imaging Station (440 CF, Rochester, NY).

Statistical analysis

Values from three or more experiments performed on different batches of oocytes/eggs were analyzed by the Student's *t*-test, Chi-squared test or one-way ANOVA, as appropriate. Differences were considered significant at $P<0.05$. Values are presented as means \pm s.e.m. Significance among groups or treatments is denoted in bar graphs by different superscripts or by the presence of asterisks.

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Author contributions

T.W. designed most experiments and performed nearly all experiments. He wrote the first draft of the manuscript and prepared all figures. N.Z. performed the thimerosal experiments (Fig. 3B), oligomycin experiments (Fig. 5B) and SERCA2b-GFP with colcemid (fig. 7D). P.V. provided the SERCA antibody and SERCA (and SERCA-GFP) plasmid vector. He participated in discussion of data interpretation and edited the manuscript. R.A.F. designed a few of the experiments, organized the manuscript and edited the numerous versions of the manuscript.

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Movie 1. Time-lapse images of SERCA2b-EGFP during oocyte maturation were obtained every 10 minutes for 12 hours.



Movie 2. cIP₃-induced Ca²⁺ release in oocytes was compared between control (upper two oocytes) and SERCA2b overexpression (lower two oocytes). Fluo-4 emitted light was imaged every 5 seconds for 4 minutes.